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Plasma glutathione suggests oxidative stress is equally present in early and late onset bipolar disorder

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Abstract:

Objectives: We previously demonstrated oxidative stress in bipolar patients and a relationship between the age of illness onset and glutathione, a principal antioxidant. In this study, we sought to replicate these findings in a new cohort of patients.

Methods: We recruited bipolar patients from the OXTEXT cohort, Warneford Hospital, Oxford, UK, of similar age and grouped them according to age of onset of illness. The early onset group comprised patients with onset <23 years, and the late group > 30 years. A third group, comprising age-matched healthy volunteers, was also included. Reduced and oxidised glutathione, cysteine, and cystine were determined in plasma, using high performance liquid chromatography. Mitochondrial DNA copy number, measured in whole blood, was also compared between patients and healthy controls.

Results: Significant increases in oxidative stress were observed in the patient groups, compared with the control group; however, no differences in glutathione-related oxidative stress measures were detected between the early and late onset bipolar patient groups. No differences were observed in the amount of mitochondrial DNA, and there was no correlation with mood state.

Conclusion: Using a more accurate method to quantify oxidative stress than in our previous study, we show that oxidative stress is a consistent feature of bipolar disorder. Although we did not reproduce our finding correlating age of onset of illness to oxidative stress, we have shown, once again, that oxidative stress is a consistent feature of bipolar disorder.

Keywords: Bipolar disorder, oxidative stress, glutathione, plasma redox state

Introduction

We previously demonstrated oxidative stress in euthymic patients with bipolar I and II disorder (1). We revealed that compared to healthy controls, bipolar patients had reduced total glutathione in peripheral blood and increased levels of oxidation. We also unexpectedly found a relationship between age of onset of illness and increased oxidative stress. Our data suggested that people with a later onset had higher levels of oxidative stress, as determined by measuring glutathione. We had expected the reverse might be more likely, and that length of illness might then, more plausibly, determine levels of oxidative stress. Our opposite finding suggested that oxidative stress might instead, be a risk factor for later onset of bipolar disorder.

In this study, we wanted to replicate our original finding in a different cohort of patients, and with a more accurate method of determining glutathione and its plasma redox state. Our previous method was the glutathione recycling assay (2), which cannot differentiate between sulfhydryl's of cysteines, glutathione, and other small thiol molecules. Therefore, the signal we observed was not uniquely due to glutathione, but also to other low molecular weight thiols. In this new study, we used a superior, more accurate high performance liquid chromatography (HPLC) method (3), that is capable of separating glutathione from other thiols like cysteine and cystine which are present in high concentrations in plasma. Crucially, this method also allows us to quantify the plasma glutathione 'redox state' more accurately, as different redox pools exist, which are not necessarily in equilibrium (4).

Oxidative stress is detrimental to biological molecules in the cell. Free radical production due to oxidative stress causes mitochondrial damage in many different pathologies, such as cardiovascular disease (5) and neurodegenerative diseases such as Parkinson's disease (6,7). More recently, studies have shown that in unipolar depression the amount of mtDNA increases with stress (8). As there is an intimate link between mitochondria and oxidative stress (9,10) and some studies have shown alterations in mtDNA but without any oxidative stress measure (11,12), we also compared mtDNA copy number between bipolar patients and control subjects.

For this study, we recruited a new cohort of bipolar I and II patients, and divided them prospectively according to age of disease onset. We hypothesised that we would again see an increase in plasma glutathione redox state, indicative of oxidative stress, in bipolar patients compared to healthy controls, and we would confirm that the highest levels of oxidative stress occurred in late-onset bipolar patients as we had observed before.

Methods

Ethics

The study was approved by the local research ethics committee (NRES Committee South Central - Oxford A; Ref 10/H0604/13) and was in accordance with the Helsinki Declaration of 1975. All participants provided written informed consent before entering the study.

Participants

Bipolar patients who fulfilled the Diagnostic and Statistical Manual of Mental Disorders, (DSM-IV) (13) criteria for bipolar I or bipolar II disorder were recruited from the OXTEXT database, Warneford Hospital, Oxford, UK. Patients were assessed using the Structured Clinical Interview for DSM disorders (SCID) (14), and the diagnosis was confirmed by an experienced psychiatrist. Euthymic patients were divided into two groups, based on age of onset of illness. The early onset cohort comprised patients (14 BP I and 12 BP II; N=26) who had a mean (\pm SD) age of onset of 18.3 (\pm 2.9) years and the late group (14 BP I and 6 BP II; N = 20) who had a mean (\pm SD) age of onset of illness 38.8 (\pm 7.5) years. There was no overlap between the two groups. Self-reported mood scores, socio-demographic and clinical data were collected from the patients as described previously (1). Age and BMI matched healthy controls (n = 29) were also recruited within the same geographical area using print and online posters. Control participants were also administered the SCID to rule out any history of axis I psychiatric disorders. Exclusion criteria for all groups included pregnancy and/or lactation.

Blood collection and processing

Blood was collected in 2 x 5 mL BD vacutainer lined with ethylenediaminetetra-acetic acid (EDTA) (# 367525, BD, USA) at 12 pm \pm 1 hour to minimise circadian variations. One tube of whole blood was frozen immediately and used at a later date to carry out mitochondrial DNA quantification. The sample processing for glutathione (reduced plus oxidised) cysteine and cystine levels was carried out as described in Jones et al 1998 (3) and in 2009 (15).

Briefly, from the second tube, a specified volume of blood was withdrawn within 2 min of collection and added to an Eppendorf tube (A) containing a 'preservation solution' (15) and centrifuged at 9800 g, room temperature for 30 s to separate the plasma. If there were any signs of haemolysis, the sample was discarded. The plasma supernatant was removed and placed in a separate Eppendorf tube (B) containing an internal standard (γ -glutamylglutamate) in an acid solution. The 'B' tubes were stored at -80°C until they were shipped to Emory University, Atlanta, USA where they were analysed in the laboratory of Professor Dean Jones using a HPLC method (3,15). Extraction and quantification of mitochondrial DNA (mtDNA) was carried out as described in Cai et al 2015 (8) using a quantitative polymerase chain reaction (qPCR) based assay. All experimental analyses were carried out under blinded conditions by someone who did not know the group designations.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 6 (GraphPad, California, USA). Data were tested for normality with the D'Agostino and Pearson omnibus normality test to determine the appropriate subsequent statistical test. Outliers were determined using Rout's test and excluded. Determination of statistical significance was carried out using a one-way analysis of variance (ANOVA) with the Holm-Sidak correction for multiple comparisons. All *p*-values have been corrected for multiple comparisons. In some cases, as deemed appropriate, two-tailed unpaired t-tests have been used to determine statistical significance. Pearson's and Spearman's product-moment correlations were conducted to determine relationships between variables for normally distributed and non-normally distributed data, respectively.

Results

Demographics

The participant's demographic and clinical characteristics are summarised in Table 1. There were no significant differences in age and BMI measures between the three groups.

Glutathione

A significant increase was observed in oxidised glutathione levels (GSSG, Figure 1B) between controls and both patient groups ($p = 0.0012$ and 0.035 for controls versus early

onset patients, and controls versus late onset patients, respectively). No significant difference in oxidised glutathione was observed between the early and late onset patient groups. Additionally, no significant differences between the three groups were observed in the reduced form (Fig 1A) or in total plasma glutathione (Fig 1E).

The structure shown in Fig. 1C is a reduced glutathione molecule. On oxidation two such molecules form a disulphide bond to create one molecule of oxidised glutathione (structure shown in Fig 1D).

None of the measured biochemical parameters differed between the early-onset and late-onset bipolar patient groups (Figure 1A, B E, F).

Glutathione redox state

A precise measure of the oxidative state of a physiological system, that considers both reducing and oxidising components, is the redox potential. It can be calculated from the Nernst equation using the measured absolute reduced and oxidised glutathione values (15,16). Based on this redox potential, there were no difference between the early and late onset patients (Figure 1F; mean \pm SD; -117.7 \pm 7.2 mV versus -118.4 \pm 8.6 mV, respectively). However, as a single group, bipolar patients had an overall more positive (or oxidising) redox state, compared to healthy controls (mean \pm SD, -118.0 \pm 7.8 mV versus -123.8 mV \pm 7.1 mV, respectively; $p=0.002$ using an unpaired t-test).

Glutathione correlations with age on onset

No significant correlations were observed between the age of onset of bipolar disorder and reduced glutathione (Figure 2A), oxidised glutathione (Figure 2B), total glutathione (Figure 2C) and glutathione redox state (Figure 2D).

Cysteine and cystine

We found no differences in cysteine (Cys) (Fig. 3A), cystine (Cyss) (Fig. 3B), total (Fig. 3E) and cysteine-cystine redox state (Fig. 3F). As in the case of glutathione, two cysteine molecules (structure shown in Fig. 3C) are oxidised and bond via a disulfide to form one molecule of cystine (structure shown in Fig. 3D).

Quantification of mtDNA

We observed no significant differences in mtDNA copy number between controls and patients, nor between the early and late onset bipolar patients (Figure 4). This indicates that despite the increase in oxidative stress seen in patients with bipolar disorder, it was not paralleled by an increase in mtDNA in this cohort.

BMI correlates positively with Cystine/Cysteine (Cyss/Cys) redox state in healthy controls, but not patients (see supplement).

In healthy volunteers, a positive correlation was observed between the Cyss/Cys redox state (Supplementary figure 1A), but no correlation was observed in either of the patient groups (Supplementary figure 1B and 1C).

Patients treated with and without lamotrigine showed no significant differences in oxidative stress.

No significant differences were observed in the plasma glutathione redox state between those patients on lamotrigine, versus those not on the drug using an unpaired t-test (Supplementary figure 2).

Discussion

Our results again demonstrate that there is a significant increase in oxidative stress in bipolar patients, compared to matched controls (1,17,18). In this study, the difference is driven by an increase in oxidised glutathione, rather than a reduction in reduced glutathione. The change in redox potential of glutathione is the same as described in our previous study. In contrast, we did not replicate our earlier finding of a relationship between oxidative stress and age of illness onset. There was no evidence for a change in the redox potential of cysteine or evidence of change in the quantity of mitochondrial DNA in the patient groups, compared with controls.

Oxidative stress is purported to play a pathological role in the development and neuroprogression of diseases such as schizophrenia and bipolar disorder (19). However, its accurate measurement is far from trivial. Indeed, it is hard to compare the extent and state of oxidative stress across different populations because laboratories utilise different indicators

and assays for quantification. First, immediate processing of blood and very careful handling is required to avoid erroneous results. Second, the related thiols cysteine and cystine form a plasma pool that is approximately 25 times larger than the glutathione pool in healthy people and so must be separated from it (3,20). To obtain the most accurate measure of the oxidative state of the body accordingly requires a specific and accurate assay; HPLC, as employed here, is state-of-the-art.

We divided the patient groups into early and late onset, matching for age. However, no differences were observed in any of the measures between the two groups. Thus, we have shown no replicated correlations between clinical measures and oxidative stress in either study. The absence of correlations with current mood (data not shown) is also notable. All patients were euthymic and on medication at the time of the study, so it is feasible that glutathione-related oxidative stress might yet be a 'state'-related biomarker. We have simply not made measurements during acute mania or depression. Similar considerations may apply to the absence of effect on mtDNA; other studies have suggested that the changes in mtDNA were partially reversible, and reflective of a stressful environment or childhood abuse (8). Moreover, it is unclear how this measure might relate to bipolar disorder yet, as some studies have found changes (12,21) whereas others have not (22). Mitochondria are certainly more sensitive to an oxidising environment (compared to other organelles) as they exist in the most reducing state compared to other organelles (23) and lack protective histones (21), but these subtleties may not be captured by the mtDNA measure employed here.

Curiously, the BMI showed a positive correlation with cystine/cysteine redox state, as expected, in healthy controls (24), but not in patients. This data (Supplementary Figure 1) suggests that there is a disruption of 'normal' metabolic processes in bipolar patients and may be related to patient medication or disease itself, and warrants more rigorous testing in a future study. Additionally, although some have purported that lamotrigine's therapeutic effects might relate to its antioxidant capabilities (25), we do not find any differences in oxidative stress in patients treated with lamotrigine, versus those who were not (Supplementary Figure 2). One caveat though, is that the group numbers are slightly skewed as the latter group had 20 additional patients.

The absence of a group difference in total glutathione is relevant to studies that have used magnetic resonance spectroscopy to measure changes in brain glutathione (26). Spectroscopy

measures only total glutathione in the target tissue of interest; our findings here show that changes in total glutathione do not necessarily reflect changes in oxidative state. Small changes in oxidised glutathione, not assessable by spectroscopy, are far better predictors of oxidative stress. Moreover, the redox state of a cell and tissue is directly related to the plasma concentrations of oxidised and reduced glutathione. Essentially, all organs communicate with one another regarding their oxidative status through the blood thiol redox state. Therefore, plasma glutathione redox is a better indicator of brain oxidative stress than total brain glutathione. We suggest that combining a plasma measure of glutathione along with brain spectroscopy might add sensitivity.

Some limitations of the study include a modest sample size, and the heterogeneous nature of the bipolar illness course, which is difficult to measure with accepted metrics. We included both BP I and BP II patients because the distinction between them is arbitrary (reflecting the severity of the manic pole of the illness) and in euthymia we see no clinical differences between the two (27). Indeed, we observed no significant difference in glutathione redox state between BP I and II patient groups (Supplementary figure 3).

In conclusion, we show that oxidative stress is increased in bipolar disorder, even when patients are euthymic. Future research should establish a unified methodology of measuring and defining oxidative stress so that they are comparable. Additionally, it might be informative to assess these measures when patients are in extreme mood states, or have not yet been prescribed medication, to ascertain how oxidative stress might be a biomarker of disease prognosis.

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Legends

Table 1: Demographic parameters and medication history (mean \pm SD or %) and for all the participants is shown. No significant difference in age and BMI was observed using a One-way ANOVA between the three groups; healthy controls, early and late onset bipolar patients.

Figure 1. Scatterplots show measured plasma reduced (A) and oxidised (B) glutathione levels, total glutathione (E) and glutathione redox state (F) levels in control subjects (black) N=29, early (blue) N=26, and late onset (red) N=20 bipolar patients. A more positive redox state is indicative of higher oxidative stress. Corrected *p* values are shown only in case where statistical significance was achieved using a one-way ANOVA with a Holm-Sidak post-test. C and D shows the structures of reduced glutathione (GSH) and oxidised glutathione (GSSG), respectively. Two molecules of GSH get oxidised at the sulfhydryl group and form one molecule of GSSG.

Figure 2. Scatterplots showing the correlation between age of onset in years (x-axis) and reduced glutathione (A), oxidised glutathione (B), total glutathione (C) and glutathione redox state (D) (y-axis) in bipolar patients. Correlations were carried out using the Pearson's correlation for normally distributed data, or the Spearman's rank correlation for data that was

not normally distributed. Both r and p values are presented on the plots. None of the measured parameters correlated significantly with age of onset.

Figure 3. Scatterplots show measured plasma levels of cysteine (Cys) (A), cystine (Cyss) (B), total cysteine and cystine (E), and Cyss/Cys redox state (F), in control subjects (black) $N=29$, early (blue) $N=26$, and late onset (red) $N=20$ bipolar patients. C and D shows the structures of reduced Cys and oxidised Cyss, respectively. Two molecules of Cys get oxidised at the sulfhydryl group and form one molecule of Cyss. No significant differences in cysteine, cystine, total cysteine+cysteine or Cyss/Cys redox state, were observed between in the three groups using an ANOVA with a Holm-Sidak correction for multiple comparisons.

Figure 4. Scatterplots showing the relative amount of mtDNA on the y-axis, for healthy controls (black), early onset (blue), and late onset (red) bipolar patients. Data was analysed using a one-way ANOVA and no significant differences were observed.

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